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A class act: conservation of homeodomain protein functions

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SUMMARY

Dramatic successes in identifying vertebrate homeobox genes closely related to their insect relatives have led to the recognition of classes within the homeodomain superfamily. To what extent are the homeodomain protein classes dedicated to specific functions during development? Although information on vertebrate gene functions is limited, existing evidence from mice and nematodes clearly supports conservation of function for the Hox genes. Less compelling, but still remarkable, is the conservation of other homeobox gene classes and of regulators of homeotic gene expression and function. It is too soon to say whether the cases of conservation are unique and exceptional, or the beginning of a profoundly unified view of gene regulation in animal development. In any case, new questions are raised by the data: how can the differences between mammals and insects be compatible with conservation of homeobox gene function? Did the evolution of animal form involve a proliferation of new homeodomain proteins, new modes of regulation of existing gene types, or new relationships with target genes, or is evolutionary change largely the province of other classes of genes? In this review, we summarize what is known about conservation of homeobox gene function.

Key words: homeobox, homeodomain, homeotic, Hox, conserved, evolution

INTRODUCTION

We celebrate this year two anniversaries. A century has passed since Bateson described and named homeotic mutations (Bateson, 1894). A decade has passed since the homeobox was discovered (McGinnis et al., 1984; Scott and Weiner, 1984). The protein fragment encoded by the homeobox, the DNA-binding homeodomain, is now viewed as a hallmark of transcription factors which control development in organisms as diverse as yeast, plants, insects and mammals.

The remarkable conservation of protein structures among developmental regulators is now so well established that apparent cases of lack of conservation are viewed with skepticism. Does conserved structure mean conserved function? The examples given below strongly suggest in several cases that homeodomain proteins of certain classes became dedicated to particular functions long ago and have maintained their dedication in ways that we do not fully understand. The homeodomain proteins, particularly the HOM/Hox group, remain the most dramatic example of retention of both protein structure and function during the evolution of developmental processes. Yet animals have enormous variety both in final form and how they develop. Attention therefore turns to learning how universal classes of developmental regulators give rise to diversity of form. In this review we focus on known or potentially conserved functions of homeodomain proteins and their regulators and targets.

HOMEODOMAINS ARE MEMBERS OF A STRUCTURAL SUPERFAMILY

Two crystal structures of homeodomains bound to DNA, and one NMR solution structure, have revealed a conserved structure for proteins sharing only 25% amino acid identity, or 15 amino acids of 60 (Qian et al., 1989; Kissinger et al., 1990; Wolberger et al., 1991). Because most homeodomains are more similar than this, the presently recognized homeodomains almost certainly have nearly identical backbone structures. The three alpha helical parts of the homeodomain create an internal hydrophobic core. One of these helices inserts into the major groove of the DNA and makes sequence-specific contacts. The N terminus of the homeodomain makes contact with the minor groove and stabilizes the association with DNA.

When we reviewed the extant 82 homeodomain sequences in 1989 (Scott et al., 1989), four amino acids were found to be diagnostic for homeodomains. The only exception was one of the yeast MAT proteins, which had three of the four relevant residues and a conservative change in the fourth. The definition has been useful, in that no protein clearly outside the homeodomain group has been found to have the critical four residues. However, the definition is arbitrary and reflects our limited ability to infer protein structure from primary sequences. Now there is evidence for an extended family of proteins which use an alpha helix to contact DNA in the major groove (Steitz, 1990; Pabo and Sauer, 1992; Schwabe and Travers, 1993).

Homeodomains are related in structure to helix-turn-helix proteins of bacteria (Laughon and Scott, 1984 ; Qian et al., 1989; Kissinger et al., 1990) and also to the POU-specific domain which is found in a family of proteins adjacent to a characteristic POU type of homeodomain. The POU-specific domain is astonishingly similar to the DNA-binding domain of the cI repressor protein of bacteriophage lambda (Assa-Munt

et al., 1993). POU proteins therefore have two DNA-binding domains, each of which is similar to helix-turn-helix proteins. The structure of a member of a third group of proteins, the *forkhead/HNF3* group, reveals yet another set of relationships. The DNA-binding forkhead domain is most similar to eukaryotic histone H5 and to the catabolite activator protein (CAP) of *E. coli.* (Clark et al., 1993a) and is therefore yet another variation on helix-turn-helix. All of the proteins in the family use a single alpha helix to make the major groove contacts with DNA, but use somewhat different framework structures to form the rest of the domain. The three groups of proteins, homeodomain, POU and forkhead, are related at the structural level without the primary sequence being discernably similar. As more protein structures are determined, the family may expand. The structural relationships between the different DNA-binding domains make it difficult to rigorously define separate families.

Within the homeodomain group different classes can be defined based on primary sequence and these classes are remarkably distinctive in their functions; those functions are, in at least some cases, conserved across vast evolutionary distances. Representative sequences of a variety of classes have been described in Rubenstein and Puelles (1994) and in Duboule (1994).

FROM HEAD TO TAIL: HOX GENE ORGANIZATION

In all animals studied to date there is a cluster of homeobox genes known as HOM-C in insects and nematodes and HOX in mammals (McGinnis and Krumlauf, 1992). We will use the term HOX to refer to all such clusters (there are many homeobox genes; only those in these particular homologous clusters are called Hox genes). Key features of HOX clusters were first observed in studies of a *Drosophila* cluster, the bithorax complex (BX-C), by E. B. Lewis (Lewis, 1963). In *Drosophila* the primordial cluster appears to have split into two major parts, the second of which is the Antennapedia complex (ANT-C) whose organization and similarity to the BX-C was recognized and analyzed by T. C. Kaufman and colleagues (Kaufman et al., 1980). Consistent with the idea of a single primordial cluster, the flour beetle *Tribolium* contains a single complex of homeotic genes (Beeman et al., 1989) as does the chordate *Amphioxus* (P. W. H. Holland, personal communication). The nematode *Caenorhabditis elegans* has a cluster composed of at least five genes (Kenyon and Wang, 1991; Wilson et al., 1994)

The organization of Hox genes in mice (and humans), *Drosophila* and *C. elegans* is summarized in Fig. 1. At least four types of Hox gene appear to have existed prior to the divergence of the ancestors of these diverse animals: the *labial, Deformed, Antennapedia* and *AbdominalB* types, each of which is represented in these animals. In addition, genes related to the fly *empty spiracles* (*ems*) or *Distal-less* (*Dll*) genes are found in or near the worm and mammalian complexes (Boncinelli et al., 1993; Wang et al., 1993). The worm *ems*-like gene is about equally similar to the *ems* and *Distal-less* (*Dll*) genes of flies, and therefore it is striking that two of the mammalian relatives of the *Dll* gene, *Dlx1* and *Dlx2*, are found near the Hoxd complex (McGuinness et al., 1992; Ozcelik et al., 1992; Simeone et al., 1994). The fly *ems* and *Dll* genes are not found in either homeotic gene cluster (Cohen et al., 1989; Dalton et al., 1989), nor are two mammalian *ems*-related genes *Emx1* and *Emx2* (Kastury et al., 1994). The presence of two *Evx* genes in the mammalian complexes (D' Esposito et al., 1991; Faiella et al., 1991) suggests still more dispersal, as the most similar fly gene, *even skipped*, is also not in either fly cluster. We are therefore left with a tentative view of an ancestral cluster containing representatives of the *lab, Dfd, Antp, AbdB, eve* and *ems* (*Dll*) types. One candidate for a seventh member of the ancestral cluster would be the genes represented in flies by *orthodenticle* (*otd*), another homeobox gene expressed in a discrete region along the anterior-posterior axis (Finkelstein et al., 1990) and in mammals by the related *Otx* genes (Simeone et al., 1992). However, the mammalian *Otx1* and *Otx2* genes do not map near any of the HOX complexes (Kastury et al., 1994).

Starting, then, from the possible ancestral cluster, what happened during the evolution of each animal type? The fly complex has been split at least once (ANT-C and BX-C) and possibly three other times (dispersion of *ems*, *eve* and *Dll*). Ironically, the fly clusters that gave rise to the mystery of how the genes are bound together may be the most dispersed of any complex yet studied. In addition, three additional homeobox genes and two other types of genes, cuticle protein genes and one encoding an immunoglobulin superfamily protein, exist in the ANT-C and may have formed by duplication or invasion, respectively (Kaufman et al., 1990). The additional homeobox genes are a pair of closely related *zen* genes, one of which is required for dorsal-ventral differentiation and the anterior-posterior maternal morphogen gene *bicoid* (*bcd*) (Berleth et al., 1988). The vertebrate protein with some similarity to *bcd* protein, *goosecoid* (Cho et al., 1991), is not known to be located near the HOX complexes. Similarly, the nematode complex is interrupted by other genes (Salser and Kenyon, 1994).

The mammalian cluster duplicated twice or three times to form four copies (Kappen et al., 1989). There are now 14 identifiable types of gene in the Hox complexes (apart from the nearby *Dlx* genes), called paralogs 1-13 and *Evx*. Paralog grouping is based on homeodomain sequence similarities as well as position within the cluster. Sequence alignments suggest the loss of paralogs from each of the clusters as shown (Fig. 1), plus the proliferation of members of the *AbdB* (paralog 9-13) group. The *Otx* genes may also have been lost, and the Hoxb and c clusters lack *Evx* representatives. The worm cluster is interrupted by other genes, like the fly cluster, and lacks any known representatives of the *eve* type.

The intriguing relationship between expression of Hox and HOM genes along the anteroposterior axis and their order along the chromosomes to which they map has been termed 'colinearity' and suggests a connection between structure of the HOM/Hox complexes and function of the genes within them. Perhaps this relationship reflects a requirement to position homeotic regulatory information of several genes in a well-defined order so that such information could influence more than one gene of the complex. If this were so, in addition to conservation of DNA encoding homeotic proteins, one would expect to find conservation of regulatory DNA within the HOM/Hox clusters. This has been shown in the case of the fly and mouse *Dfd* homologs, since regulatory sequences from either are able to respond to appropriate spatial cues when

introduced into the other species (Awgulewitsch and Jacobs, 1992; Malicki et al., 1992; see below).

HOX GENE FUNCTIONS

Several rules governing homeotic gene function have been fairly well conserved. (1) Genes are ordered along the chromosome in the same order as their expression and function along the anterior-posterior axis of the animal. (2) More genes are usually expressed in more posterior regions. (3) Loss of gene function leads to loss of structures or to development of anterior structures where more posterior structures should have formed. (4) Activation of genes where they should be off, i.e. gain-of-function mutations, leads to posterior structures developing where more anterior structures would normally be found. To these generalizations we may add some molecular data. (5) Each homeotic gene contains a single homeobox and encodes a sequence-specific DNA-binding protein which acts as a transcription factor. Some encode a family of proteins with alternatively spliced mRNAs. (6) Most of the homeotic genes are transcribed in the same direction, with the 5′ ends of transcription units oriented toward the posterior end of the HOX cluster.

In flies, the clustered homeotic genes of the Antennapedia and bithorax complexes determine segment identity by promoting the morphogenesis of appropriate anatomical structures within particular segmental or parasegmental domains of the body. These domains, which are reiterated units along the anterior-posterior axis, are established before the homeotic genes are active. The fly homeotic genes are not required for establishment of the segmental body plan, but only to govern segmental form. Mutations in fly homeotic genes lead to alterations in cell fate decisions, not changes in segment number. The epidermal expression patterns and sites of action are summarized in Fig. 2. A single fly homeotic protein, rather than a combination, is in some cases sufficient to activate a morphogenetic pathway. For example, ubiquitous expression during early embryogenesis of the *Ultrabithorax* homeotic gene, which normally promotes anterior abdomen identity and is transcribed primarily there, leads to specification of head and thoracic segments as anterior abdomen-like segments (Mann and Hogness, 1990). *Ubx* does not activate any other homeotic genes and represses some more anteriorly acting ones, so the *Ubx* protein is sufficient to organize pattern without other Hox proteins.

The *C. elegans* HOM-C or Hox genes (Kenyon and Wang, 1991) provide fascinating information about the effects of Hox genes on individual identified cells (reviewed in Salser and Kenyon, 1994). For example *mab-5* and *lin-39* govern migration of certain neuroblasts (Clark et al., 1993b; Salser et al., 1993); heat shock promoter activation of *mab-5* causes cells to change direction during their migration (Salser and Kenyon, 1992). The nematode genes are expressed in the order along the anterior-posterior axis expected from mouse and fly studies, even though the order of two of the nematode genes on the chromosome is inverted compared to other Hox genes (Fig. 1) (Cowing and Kenyon, 1992). Cross-regulatory interactions among the homeotic genes limit their domains of expression (Salser et al., 1993). Cell fates are controlled largely autonomously by the Hox genes (Salser and Kenyon, 1992; Clark et al., 1993b). There are clear examples of combinatorial actions of the genes, as when cells fuse in response to *mab-5* and *lin-39* but not to either alone (Salser et al., 1993). In other cases the presence of one homeotic gene activity precludes evident action of another.

The vertebrate Hox genes also instruct cells to undergo appropriate developmental decisions. Hox genes are expressed in nearly every cell type, but have been most extensively studied for their roles in the developing central nervous system and axial skeleton. They are transcribed in limited regions along the anterior-posterior axis, like the fly genes, although in more substantially overlapping patterns. Fewer Hox genes are expressed in the anterior than posterior, suggesting that progressively more caudal structures may depend upon concerted actions of multiple homeotic proteins. However, the posterior prevalence model argues against this possibility (see below). The scarcity of antibody studies makes it difficult to ascertain whether multiple Hox proteins are found in the same cell, but this seems likely to be true given that this is the case in flies (Carroll et al., 1988). The picture is further complicated by the four sets of Hox genes (Fig. 1). Because it is often the case that corresponding paralogs in different Hox clusters are expressed in similar patterns, particularly for the Hoxa and Hoxd clusters, redundant or partially redundant function of the genes may make interpretation of mutations in only one gene difficult. This problem will soon be addressed by the engineering of doubly mutant transgenic mice, but in the meantime a considerable amount can be learned from single mutations. The mutations have been engineered in embryonic stem cells in culture and then introduced into the mouse germline. These mutants provide convincing evidence of homeosis and leave little doubt of the power of Hox genes to control cell fates during development.

In the central nervous system, the anterior border of expression of many of the Hox genes coincides with rhombomeric boundaries, the rhombomeres being transient, reiterated bulges of the hindbrain thought to be indicative of segmental organization of the brain. Loss-of-function mutations of *Hoxa-1* and *Hoxa-3* cause defects in hindbrain and branchial regions of the mouse, but do not appear to cause homeotic transformations of the affected regions (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Carpenter et al., 1993). The cells affected by both mutations are derived from the cranial neural crest. A reduction in the number of rhombomeres was observed in *Hoxa-1* mutants (Lufkin et al., 1991; Carpenter et al., 1993). Mutations in the two fly homeobox genes *ems* and *otd*, not located in the HOX clusters but possibly there ancestrally, cause embryos to develop with altered fates in the head and with reduced numbers of segments. It has been suggested that deletion of body parts, in contrast to homeotic transformation, occurs when the absence of one homeotic gene function does not result in the expression of another in it's place (McGinnis and Krumlauf, 1992). This idea may explain why both types of phenomena are observed in flies and in mice.

Targeted gene disruption of *Hoxc-8*, *Hoxb-4*, or *Hoxa-2*, as well as constitutive overexpression of *Hoxa-7*, *Hoxc-6*, *Hoxc-8* or *Hoxd-4*, causes homeotic transformations in mouse embryos (Kessel et al., 1990; Jegalian and De Robertis, 1992; Le Mouellic et al., 1992; Lufkin et al., 1992; Pollock et al., 1992; Gendron-Maguire et al., 1993; Ramirez-Solis et al., 1993; Rijli et al., 1993). In the *Hoxc-8* mutant, the first lumbar

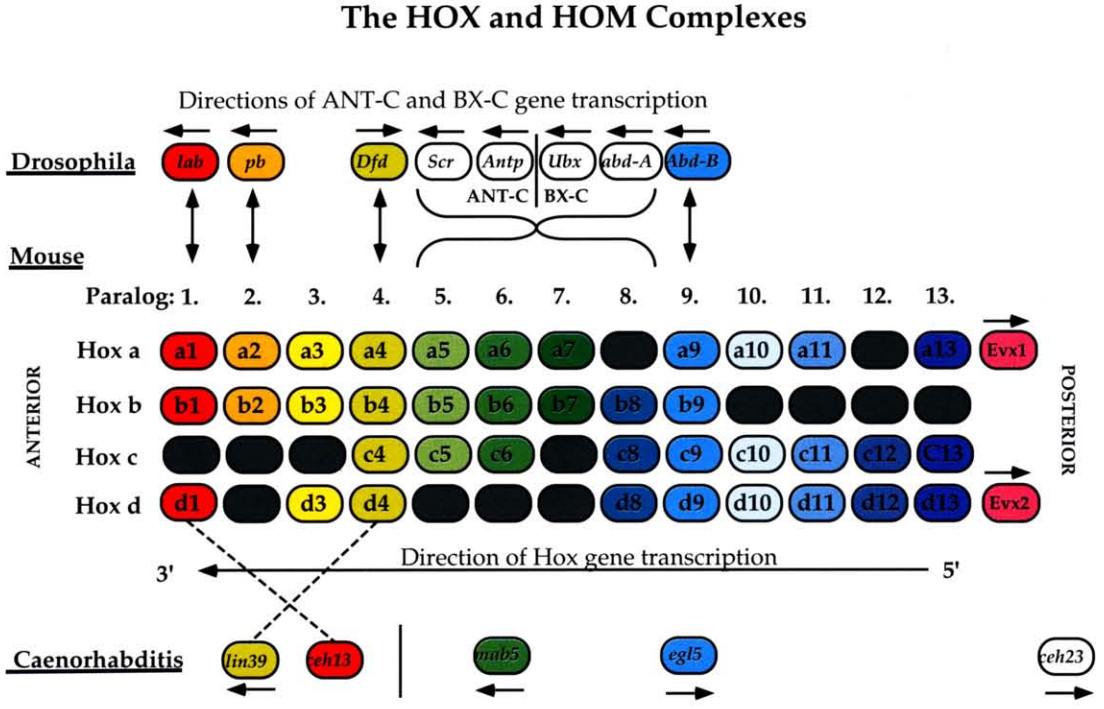

Fig. 1. Comparitive organization of Hox complexes. The alignments of the *Drosophila*, nematode and mouse complexes are shown. Colors indicate similar homeodomain sequences. The order of the genes along the chromosome is as shown, with the order roughly corresponding to where along the anterior-posterior body axis a gene is expressed or, in the case of mammals, the anterior-most limit of expression. Mammalian nomenclature is as described in Scott (1992). The four mammalian clusters are thought to have arisen by two duplication events. Categories 1-13 are called 'paralog' groups and are indicative of homeodomain sequence similarity. Gray ovals indicate the lack of a paralog from a cluster. The Evx genes encode homeodomains most closely related to the *even skipped* segmentation gene of *Drosophila*. The assignment of *Scr*, *Antp*, *Ubx* and *abd-A* to any particular paralog group is uncertain. The *ceh-23* gene, located 30 kb from *egl-5* (Wang et al., 1993) is related to the *empty spiracles* or *Distal-less* genes of flies and mammals, and its presence near the nematode Hox cluster may be indicative of the ancestral linkage of this type of gene. Two related mammalian genes have been mapped near the Hox complexes with cytological studies but the molecular distance is unknown and could be substantial (McGuinness et al., 1992; Ozcelik et al., 1992; Simeone et al., 1994). The direction of *ceh-13* transcription is unknown. See text for additional references.

vertebra is converted to a thoracic vertebra, thus producing a 14th pair of ribs (Le Mouellic et al., 1992). Mice lacking *Hoxa-2* function have anterior transformations of skeletal elements derived from the second branchial arch (Rijli et al., 1993; Gendron-Maguire et al., 1993). Two *Hoxb-4* mutations have been introduced into mice, both of which cause transformations of the second cervical vertebra from axis to atlas (Ramirez-Solis et al., 1993). All of these phenotypes are consistent with loss-of-function homeotic transformations observed in flies: posterior structures are converted to more anterior ones.

Overexpression of either *Hoxa-7* or *Hoxd-4*, analogous to gain-of-function homeotic mutations in flies, leads to transformations of anterior structures into posterior structures (Kessel et al., 1990; Lufkin et al., 1992). *Hoxd-4* overexpression transforms occipital bones into structures that resemble cervical vertebrae whereas *Hoxa-7* overexpression transforms the basioccipital bone into a proatlas structure. Overexpression of *Hoxa-4* causes a condition similar to congenital megacolon, probably due to abnormalities in the enteric nervous system (Tennyson et al., 1993). Although this gene is expressed in a variety of tissues including spinal cord, ganglia and gut mesoderm, abnormalities were only observed in the terminal colon.

Mice that overexpress *Hoxc-6* (Jegalian and De Robertis, 1992), or *Hoxc-8* (Pollock et al., 1992), develop rib-bearing vertebrae in lieu of one (or more) of the lumbar vertebrae, a transformation of posterior to anterior similar to the *Hoxc-8* loss-of-function phenotype (Le Mouellic et al., 1992). Because *Hoxc-8* is normally expressed in that region, the *Hoxc-8* overexpression phenotype is presumably due to heightened or temporally incorrect expression. More work is needed to understand the origins of these effects, but the observed phenotypes could be partially explained if overexpression of *Hoxc-6* or *Hoxc-8* blocks function of *Hoxc-8* in its normal domain of expression.

A hierarchy of homeotic protein function exists amongst homeotic genes. Most fly HOM genes are unable to transform the identity of segments posterior to their normal domains of expression when activated ubiquitously, even though they are capable of transforming anterior segments (Gonzales-Reyes and Morata, 1990; Gonzales-Reyes et al., 1990; Mann and Hogness, 1990). There are exceptions: either *Antp* or *Scr* can prevail in the thorax (Gibson et al., 1990). Since the inability to transform posterior regions is dependent on posterior homeotic protein function, more posterior-acting homeotic proteins may be 'dominant' with respect to function over more anterior-acting proteins. This phenomenon has been termed

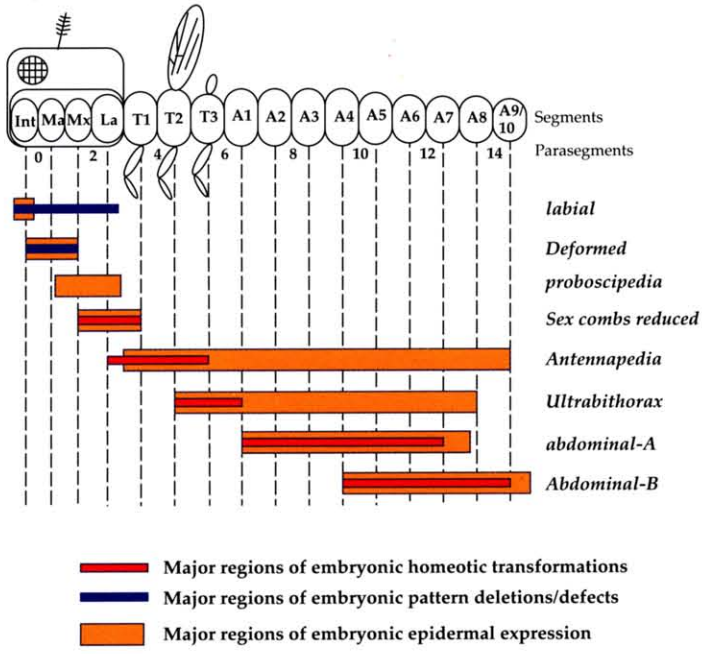

Fig. 2. Expression and function of the *Drosophila* HOM genes. Fly body segments include the intercalary (Int), mandibular (Ma) and labial (La) segments of the presumptive head, T1 to T3 segments of the presumptive thorax, and A1 to A10 segments of the presumptive abdomen. Normal expression patterns of *labial* (*lab*; Diederich et al., 1989), *Deformed* (*Dfd*; Jack et al., 1988; Mahaffey et al., 1989), *proboscipedia* (*pb*; Pultz et al., 1988; Mahaffey et al., 1989), *Sex combs reduced* (*Scr*; (Riley et al., 1987; Pattatucci and Kaufman, 1991), *Antennapedia* (*Antp*; Carroll et al., 1986; Wirz et al., 1986), *Ultrabithorax* (*Ubx*; Beachy et al., 1985; White and Wilcox, 1985; Carroll et al., 1988), *abdominal-A* (*abd-A*; Karch et al., 1990) and *Abdominal-B* (*Abd-B*; Celniker et al., 1989; DeLorenzi and Bienz, 1990) are indicated. Mutations in *Scr*, *Antp*, *Ubx*, *abd-A* and *Abd-B* produce homeotic transformations in the embryo whereas mutations in *lab* and *Dfd* produce pattern deletions. Alterations in mandibular, maxillary and labial segments in *lab* mutants may be due to the secondary effect of failure of head involution. *pb* is expressed in the embryo but no functions for it have been detected there. *pb* is located between *lab* and *Dfd* in the ANT-C complex.

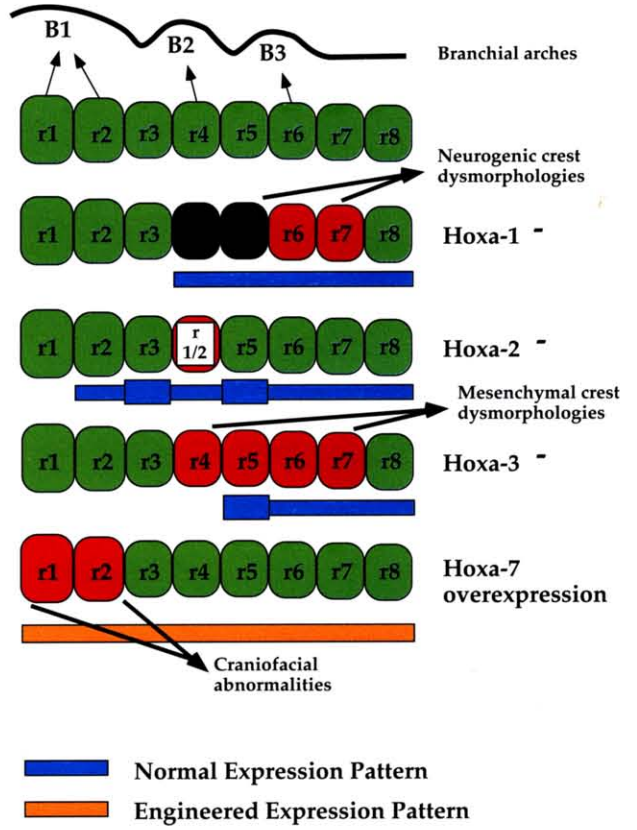

Fig. 3. Mouse Hox mutations affect cranial development. Rhombomeres 1 to 8 (r1-r8) are thought to represent segmental organization of the hindbrain. The first three branchial arches (b1-b3) are derived from neural crest cells which originate from specific rhombomeres (arrows) and produce head mesodermal structures such as bone and connective tissue. *Hoxa-1* mutations affect neurogenic crest-derived structures such as sensory and motor ganglia from the region encompassing rhombomeres 4 to 7. *Hoxa-3* mutations also affect this region of the hindbrain; however, dysmorphologies are specifically observed for mesenchymal crest-derived structures. For example, both the thymus and parathyroid glands fail to develop. Mice deficient in *Hoxa-2* show homeotic transformations of 2nd branchial arch derivatives to 1st branchial arch derivatives. Reichert's cartilage, which forms the stapes bone of the middle ear as well as other structures, is replaced with Meckel's cartilage, which forms the malleus and incus. *Hoxa-7* overexpression throughout the hindbrain produces craniofacial abnormalities such as cleft palate, open eyes and non-fused pinnae. These structures are derived from 1st branchial arch neural crest cells. *Hoxa-7* neural expression is normally confined to the spinal cord. For references, see text. Normal expression patterns are indicated, when appropriate, for *Hoxa-1* (Murphy and Hill, 1991), *Hoxa-2* (Hunt et al., 1991; Tan et al., 1992), *Hoxa-3* (Gaunt, 1988; Gaunt et al., 1988) and *Hoxa-7* (Mahon et al., 1988). See Krumlauf et al. (1993) for a review. Rhombomeres indicated by red color are affected by the indicated mutation. Rhombomeres indicated in black are deleted in the specified mutant. The anterior homeotic transformation of skeletal elements derived from the second branchial arch to structures normally derived from the first branchial arch (thus adopting fates of neural crest cells emanating from rhombomeres 1 and 2) in *Hoxa-2* mutants is indicated by the white box.

'phenotypic suppression' (Gonzales-Reyes and Morata, 1990). A strikingly similar phenomenon is observed in mice. In general, mice mutant for Hox genes show homeotic transformations in the anteriormost region where that Hox gene is normally expressed and not within regions where a more posterior Hox gene is expressed (see Figs 4 and 5). Therefore, the more posterior Hox genes are able to promote the appropriate morphological responses without the assistance of more anterior genes (termed 'posterior prevalence' by Duboule, 1991). Consistent with these results, overexpression of two Hox genes in the mouse leads to posteriorization of anterior structures (Kessel et al., 1990; Lufkin et al., 1992); however, this is not the case for two other Hox genes, where overexpression leads to anteriorization in posterior regions (Fig. 5; Jegalian and De Robertis, 1992; Pollock et al., 1992). In the latter two cases levels or timing of expression may lead to the different outcome.

Elegant experiments by the McGinnis and Morata groups have demonstrated a functional relationship between the fly HOM and vertebrate Hox genes (Malicki et al., 1990; McGinnis et al., 1990; Bachiller et al., 1994). Ubiquitous

expression of mouse *Hoxb-6*, most closely related to the fly *Antp* gene, causes homeotic transformations in embryos that are similar in nature to those produced by ectopic expression of *Antp* (Malicki et al., 1990). In addition, antenna-to-leg transformations, the classic *Antp* gain-of-function phenotype, are observed in the adult head when *Hoxb-6* is ubiquitously expressed in larvae. Along the same lines, ubiquitous expression of the *Dfd* human homolog *Hoxd-4* can provide some functions attributed to *Dfd* such as autoactivation of the *Dfd* gene in embryos and larvae, producing phenotypic alterations of adult head structures similar to those observed with a dominant allele of *Dfd* (McGinnis et al., 1990).

Ubiquitous expression of several mouse Hoxd genes in flies (*Hoxd-8* to *Hoxd-11*) suppresses fly homeotic gene function, but in a most intriguing way (Bachiller et al., 1994). *Hoxd-8* is most closely related to the *Antp/Ubx/abd-A* class of homeobox genes whereas *Hoxd-9* to *Hoxd-11* are most closely related to the *Abd-B* gene. *Hoxd-8* to *Hoxd-11* are sequentially arranged along the chromosome 3′ to 5′ and act in progressively more posterior regions in the mouse. When expressed in flies, the more posterior acting Hox genes are better able to overcome the effects of the clustered fly homeotic genes than the anterior-acting genes. The result of such experiments is the transformation of affected segments to a thoracic ground-state character, even though endogenous fly homeotic genes in these regions are expressed at slightly reduced to normal levels (Bachiller et al., 1994). Thus, *Hoxd-8* or *Hoxd-9* prevails over head-specific homeotic genes, *Hoxd-10* prevails over head- and thorax-specific homeotic genes, and *Hoxd-11* prevails over head-, thorax- and abdominal-specific homeotic genes. In addition, *Hoxd-11* can activate the endogenous *Abd-B* gene as well as an *Abd-B* target *(empty spiracles)* even in the absence of endogenous *Abd-B*. Filzkorper, morphological readouts of *Abd-B* function, are ectopically induced by *Hoxd-11* in these experiments. Even though *Hoxd-9* to *Hoxd-11* all show an equal degree of similarity with respect to *Abd-B*, they still differ in terms of their ability to suppress the fly homeotics. Thus, the posterior prevalence rule holds: the farther back a mammalian gene is expressed, the better it is at overriding more anterior homeotic genes.

Most of the genes discussed so far act in the trunk of the animal. Additional Hox genes, and homeobox genes not presently found in all Hox complexes, act primarily in head development. At least some of these head homeobox genes may have once been located in a primordial homeotic complex. As mentioned previously, the vertebrate and nematode complexes provide evidence for an original Hox complex with more types of genes than the present fly complex. The head genes may have functions analogous to those of the Hox genes acting in the trunk, but the complexities of anterior structures make the regulatory roles of these genes less clear.

HOMEOBOX GENE FUNCTIONS IN THE ANTERIOR EMBRYO: EVIDENCE FOR MORE DIVERSE ORIGINAL HOX CLUSTERS

The Hox cluster genes *lab*, *Dfd* and *Scr* contribute to fly head patterning (Fig. 2). However none of the Hox genes 'cover' the most anterior parts of the embryo. The most anterior cells employ other genes, including at least three types of fly homeobox genes (*Dll, otd* and *ems*) not present in the Hox cluster (Cohen and Jürgens, 1991; Finkelstein and Perrimon, 1991). *Dll* is expressed in the primordia of the limbs, in the brain and in head ectoderm, in particular the anlage of the facial sensory appendages within the labral, antennal, maxillary and labial segments (Cohen et al., 1989). *otd* and *ems* are expressed in the cephalic region of the head (Dalton et al., 1989; Finkelstein et al., 1990; Wieschaus et al., 1992), *otd* expression overlapping with *ems* but extending anterior to it. *otd* mutants have partial and complete deletions in pre-antennal and antennal segments, respectively, whereas mutants of *ems* lack antennal and intercalary segments. A smaller region of the pre-antennal segment is also deleted in *ems* mutants. Both *otd* and *ems* expression is dependent on bicoid and torso expression to define their posterior and anterior boundaries, respectively, but does not require gap or pair-rule segmentation gene input (Finkelstein and Perrimon, 1990). Based on expression patterns as well as genetic data, Cohen and Jurgens (1991) have proposed that both segmentation and segmental identity are controlled by *ems* and *otd* as well as *buttonhead* (*btd*), whose expression reaches more posteriorly than the other two. The model proposes that a segment's identity is determined by the particular combination of such genes expressed in a segment. For example, the antennal segment expresses *otd*, *ems* and *btd*, whereas the intercalary segment expresses only *ems* and *btd*.

The vertebrate homeobox genes related to the fly head genes are expressed in patterns similar to those of the fly genes, suggesting evolutionary conservation of function underlying dramatically different final morphologies. Four vertebrate genes, two homologous to *otd* (*Otx-1*, *Otx-2*) and two similar to *empty spiracles* (*Emx-1*, *Emx-2*), are primarily expressed in the anterior developing brain (Simeone et al., 1992). There are distinct overlaps in the patterns of expression of the four genes. *Otx-2* is most broadly expressed, from telencephalon to mesencephalon, inclusive. *Otx-1* expression is contained within that of *Otx-2*. Similarly,the *Emx* genes are expressed in a portion of the *Otx-1* domain and *Emx-1* is expressed within the *Emx-2* domain. In mice *Otx-2* expression appears earliest, at 7.5 days d.p.c., followed by *Otx-1* and *Emx-2* and then *Emx-1*. Thus different genes may be used for different stages of fate determination.

Six mouse *Distal-less* homologs have been isolated (Price et al., 1991). The expression patterns of four of them, *Dlx-1*, *Dlx-2*, *Dlx-5* and *Dlx-6*, have been reported (Dollé et al., 1992; Simeone et al., 1994). All four genes are expressed in the forebrain, the primordia of the face and neck (branchial arches), and the ectoderm of the limb buds, a pattern strikingly conserved from fly to mouse. In addition, *Dlx5* and *Dlx-6* are expressed in the developing skeleton after early cartilage formation (Simeone et al., 1994). The first branchial arch, which has high levels of *Dlx* expression, gives rise to the mouth/jaw region of the mouse. In flies, the maxillary, labral and labial segments, domains of *Dll* expression, also give rise to mouth structures. There is no HOX expression in forebrain regions, so perhaps the *Dlx*, *Otx* and *Emx* gene activities determine cellular identity there. The idea of a commonality of gene functions in face and limbs between insects and vertebrates is nearly beyond belief, given the utterly different terminal morphologies. However, it was not long ago that homologies now generally accepted, such as Hox gene rela-

tionships, were rightly viewed with enormous skepticism! We will simply have to wait and see what is truly conserved.

The Hox clusters remain the most remarkable example of conservation of regulatory genes involved in development. While the parallels described here are striking, the differences in Hox gene expression and function among animal types are notable as well. The major problem that we face is in trying to understand what it means for a gene to define a certain region of the body. What is in common between the thorax of a fly and a human?

POTENTIALLY CONSERVED FUNCTIONS OF OTHER CLASSES OF HOMEOBOX GENES

In addition to the clustered Hox genes, other distinctive types of homeodomain proteins appear to have evolutionarily conserved functions in development. The evidence is often weak but suggestive enough to warrant summarizing. We emphasize that many homeodomain proteins are used at different times and in different tissues, and only some of their functions may be conserved.

forkhead and *caudal* in the gut

The *fork head* domain has a structure related to that of histone H5 and the bacterial CAP protein, and is more distantly related to homeodomains (Clark et al., 1993a). Like the homeodomain proteins, proteins containing forkhead/HNF3 domains may have evolutionarily conserved functions (Clevidence et al., 1993; Hromas et al., 1993). *forkhead* (*fkh*) is required for formation of the gut endoderm in flies (Jürgens and Weigel, 1988; Weigel et al., 1989). The HNF3 proteins were discovered as factors needed for transcription of genes in mammalian liver (Lai et al., 1991). Because the forkhead class of proteins has many other functions, however (Lai et al., 1993), the apparent similarity in some of those functions may or may not be meaningful.

A second homeobox gene likely involved in gut development is represented by *caudal* in flies (Mlodzik et al., 1985; Macdonald and Struhl, 1986) and by the *Cdx* genes in vertebrates (Duprey et al., 1988; Joly et al., 1992; Frumkin et al., 1993, 1994). In flies, *cad* is required for development of posterior cuticle structures. *cad* is also expressed in the developing gut. *Cdx* genes are expressed in the intestinal epithelium and mesenchyme and may be involved in gut closure.

prospero and *cut* in the nervous system

The *prospero* 'family' of proteins is as yet represented by only two genes, but because they exist in both mammals and insects the family is probably genuine. These proteins have among the most divergent homeodomains known in higher animals. The mouse and fly proteins share both homeodomain and C-terminal protein sequences (Oliver et al., 1993). The *Drosophila pros* gene was identified as a regulator of neuron differentiation required after the completion of mitotic divisions (Doe et al., 1991; Vaessin et al., 1991). The gene is transcribed in embryonic neuroblasts and the mRNA is translated in descendant ganglion mother cells and young neurons. It is also expressed in the cone cells which secrete lens in the developing eye and in the midgut. The mammalian gene *Prox1*, which may or may not be the only mammalian version of *pros*, is also active during neural development. The similarity extends to the types of cells in which both genes are active: undifferentiated neurons, eye lens, heart, pancreas, and liver. The strongest argument for genuine conservation is rather weak: expression in neurons after cell division but prior to differentiation.

The fly gene *cut* encodes a distinctive type of homeodomain protein (Blochlinger et al., 1988). *cut* is expressed in many tissues, governing neural differentiation as well as other developmental events (Bodmer et al., 1987; Blochlinger et al., 1990; Jack et al., 1991; Liu et al., 1991). A related mammalian gene called *Clox* is expressed in diverse tissues in mice (Andres et al., 1992) including cartilage, liver, skeletal muscle, brain, lung and heart. Thus both the vertebrate and invertebrate genes act in a diverse set of tissues; what links the different tissues together in terms of *cut* function is not yet clear. Each of the two proteins contains three 73 aa repeats outside the homeodomain, the *cut* repeats.

MADS boxes and homeoboxes: the heart of the matter

The earliest known marker of vertebrate heart development is expression of the cardiac-specific homeobox gene *Csx* (Komuro and Izumo, 1993). *Csx* is expressed in the myocardium in 7.5 day mouse embryos, the late primitive streak stage when the future heart is just a small flattened plate. Thus the gene is active long before overt heart differentiation. Expression of *Csx* persists through adulthood. Although no functional studies are yet available, the great specificity of *Csx* expression strongly suggests a role in controlling heart muscle development.

A second early marker of heart development, though one that is also expressed in many other tissues, is the transcription factor MEF-2C (E. Olson, personal communication). MEF-2C is one of a family of transcription factors active in a broad range of mesodermal tissues. The protein contains a MADS box (Pollock et al., 1991), a sequence found in a larger family of proteins including serum response factor and some plant homeotic genes. The MEF group contains a MEF-specific domain adjacent to the MADS box. The best studied MADS box protein is a yeast transcription factor called MCM1. MCM1 associates with the repressor MATα2 to repress a mating type-specific genes (Johnson, 1992). The two proteins together therefore contribute to the control of yeast differentiation. It is the 70 amino acid MADS box region that binds to MATα2 and cooperates with it in binding DNA (Johnson, 1992).

A fly heart, or 'dorsal vessel', is completely different in appearance from a vertebrate heart. The embryonic heart is composed of several parallel rows of cells which later form loosely knit valves to move hemolymph through the larva without the use of ducts. Despite the drastic difference in morphology, the fly and vertebrate hearts have common genetic regulators. A MEF2-like gene, called DMEF2, has been found in flies and is expressed in the developing heart as well as in visceral mesoderm (Lilly et al., 1994). Not to be outdone, a homolog of *Csx* called *tinman* is expressed in the developing fly heart. *tinman* is expressed in both visceral mesoderm and heart earlier in embryogenesis, but comes to be restricted to the heart. Mutant animals that lack *tinman* function develop neither the visceral mesoderm nor the heart (Azpiazu and Frasch, 1993; Bodmer, 1993).

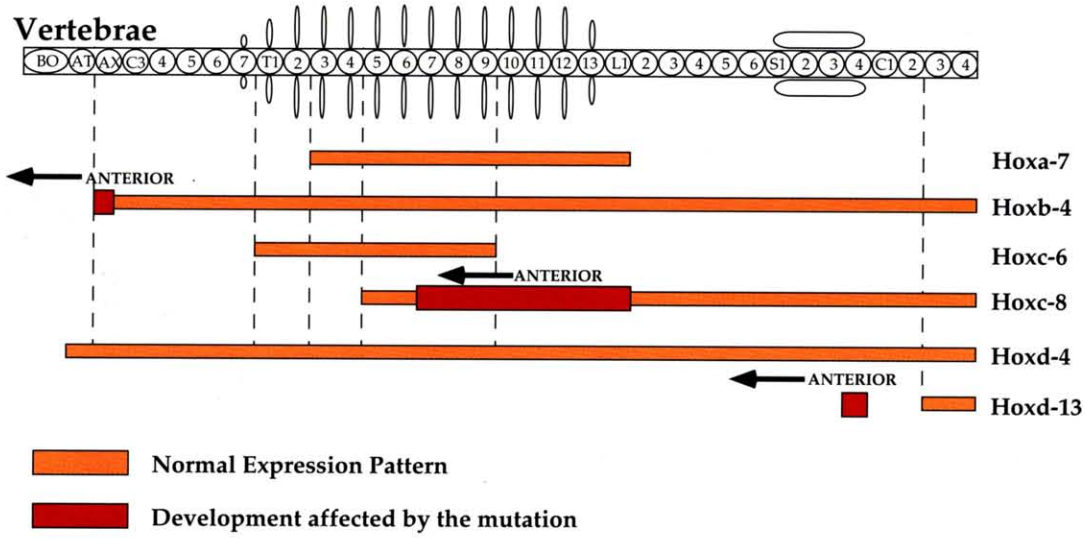

Fig. 4. Mouse Hox mutations result in anteriorly directed homeotic transformations in the paraxial mesoderm. A schematic representation of the vertebrae of the mouse is shown along with the expression patterns of several hox genes prior to vertebral specification. The basioccipital bone (BO) is connected to the cervical vertebrae (C3 through 7), which includes the atlas (AT) and axis (AX). Thirteen rib-bearing thoracic vertebrae (T1 to 13) are followed by six lumbar (L1 to 6), four sacral (S1 to 4) and four caudal (C1 to 4) vertebrae. Normal expression patterns of *Hoxa-7* (Mahon et al., 1988), *Hoxb-4* (Gaunt et al., 1989), *Hoxc-6* (Sharpe et al., 1988), *Hoxc-8* (Gaunt, 1988; Gaunt et al., 1988), *Hoxd-4* (Gaunt et al., 1989) and *Hoxd-13* (Dolle et al., 1991) are indicated, although the precise location of boundaries of expression, especially posteriorly, is unclear at this time. In general, knockouts of *Hoxb-4*, *Hoxc-8* and *Hoxd-13* affect the most anterior region in which the gene is normally expressed. The homeotic transformations which result are in the anterior direction, as observed for loss-of-function homeotic mutations in flies. Although the phenotype observed for *Hoxd-13* mutations is consistent with a homeotic transformation, the authors argue that the phenotype could be due to altered growth properties of cells instead. It is also important to note that the reported expression pattern of *Hoxd-13* does not include the region of the vertebrae that is affected in the mutation. Expression may extend further anteriorly. Alternatively, cell non-autonomous events may dictate the phenotype. See text for additional references.

The presence of both types of regulatory molecule in corresponding tissues in animals with a common ancestor some 600 million years ago suggests the dedication of the two molecules to creation of a heart-like organ in the ancestors of insects and vertebrates. The relationship is remarkable given the utter disparity in structure. The results also suggest a possible intimate relationship between the MADS and *tinman* proteins, as in the yeast homeodomain-MADS box association.

The *engrailed* group and neural development

The *engrailed* (*en*) gene in *Drosophila* is one of the few segment polarity genes that encodes a homeodomain-containing transcription factor. *En* is expressed in the posterior half of each segment in the ectoderm as well as in the developing nervous system (DiNardo et al., 1985; Kornberg et al., 1985). Flies homozygous for a special allele that allows adult survival have posterior wings similar in appearance to the anterior wing (Eker, 1929; Morata and Lawrence, 1975). Similarly, clones of cells in various adult tissues that lack *en* function exhibit posterior-to-anterior transformations of fate (Morata et al., 1983). Embryos homozygous for a null *en* allele have disruptions of pattern in each segment (Nüsslein-Volhard and Wieschaus, 1980; Kornberg, 1981). The closely linked *invected* gene is highly homologous to *en* and is expressed in a very similar or identical pattern.

En expression is initially under the control of pair-rule segmentation genes, but later comes under the influence of segment polarity genes such as *wingless*, the closest fly relative to the mammalian *Wnt-1* gene (DiNardo and O'Farrell, 1987; Ingham et al., 1988; Heemskerk et al., 1991). The gene also autoregulates. The successive waves of regulation may be typical for genes that remain active during a broad period of development and which must be responsive to changing arrangements of cells.

In addition to its role in metamere specification, *en* is expressed in elaborate and precise segmentally reiterated patterns in the developing nervous system (Doe, 1992). Like the striped expression pattern of *en* in the epidermis, the neural pattern is exquisitely conserved in arthropods from flies to crayfish (Patel et al., 1989). Neural expression seems to be a more general phenomenon than the metameric expression since no other higher organisms included in this study express *en* in developing metameres.

Two mouse genes with significant homologies to the fly *en* genes have been identified and named EN-1 and EN-2 (Joyner and Martin, 1987). The genes are expressed in a similar pattern at the midbrain-hindbrain border. *Wnt-1*, which is activated earlier in development than EN-1 or 2 and is expressed at the midbrain-hindbrain border, is required for activation of both EN genes (Bally-Cuif et al., 1992). This relationship between these vertebrate genes is reminiscent of the *en-wg* interaction in flies. The result of the *Wnt-1* mutation in mice is the absence of the entire midbrain and part of the hindbrain (McMahon et al., 1992). Thus, *Wnt-1* is required in regions where it does not appear to be expressed. Several other vertebrate *en* genes have been isolated, including 3 from zebrafish, and their expression patterns are conserved to a large extent (see (Rubenstein and Puelles, 1994) for review). Loss-of-function mutations in EN-2 result in subtle disruptions of the cerebellum (Joyner et al., 1991; Millen et al., 1994), suggesting that EN-1 may be able to partially substitute for EN-2 in the mouse to mask the full phenotype. Nonetheless, in EN-2 mutants, several features of the phenotype are worth noting: (1) all major morphological defects occur in the posterior cerebellum, and (2) cerebellar

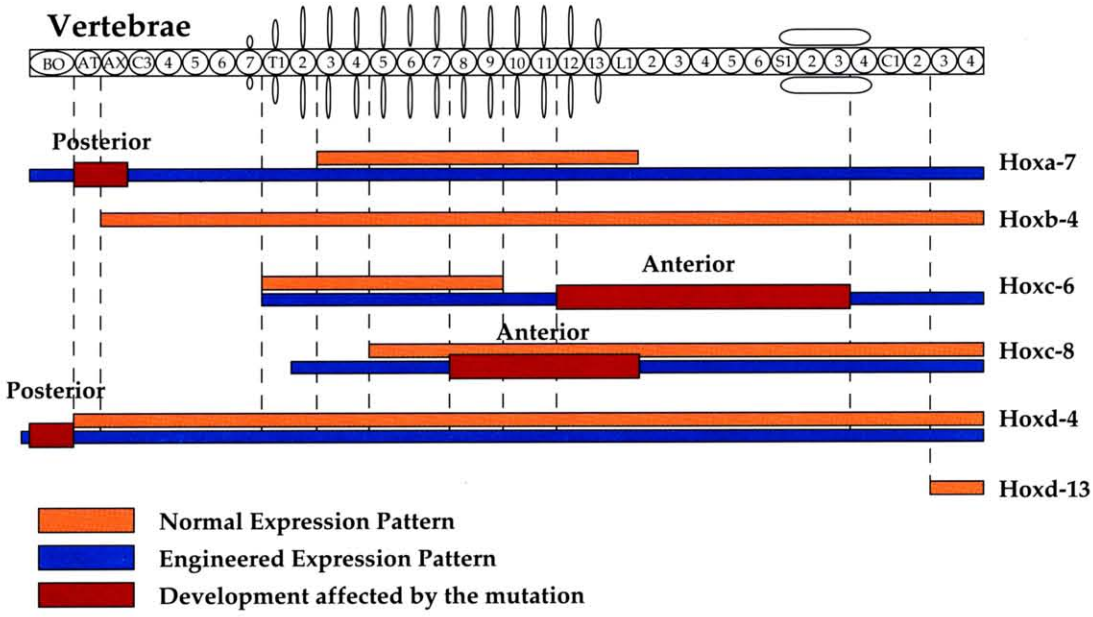

Fig. 5. Overexpression of Hox genes in the paraxial mesoderm causes homeotic transformations. A schematic representation of the vertebrae of the mouse is shown along with both normal and engineered hox expression in the paraxial mesoderm. Some Hox genes expressed anterior to their normal domains of expression cause posteriorized homeotic transformations (eg, *Hoxa-7* and *Hoxd-4* overexpressing mice) whereas other Hox genes expressed posterior to their normal domains of expression or at elevated levels cause anteriorized homeotic transformations (eg, *Hoxc-6* and *Hoxc-8*). Results obtained with *Hoxa-7* and *Hoxd-4* overexpressing mice are consistent with the posterior prevalence model whereas results from *Hoxc-6* and *Hoxc-8* overexpressing mice are not (see text).

lobe identities appear to be changed. Two of the lobes are transformed from posterior to anterior identities whereas one lobe is transformed from an anterior to posterior identity. Thus, EN-2 subdivides the brain in vertebrates, as *en* subdivides *Drosophila* segments, and plays a role in cell fate determination. In fact, the fly *en* has been described both as a segmentation gene and as a homeotic gene.

Analyses of flies mutant for either of two segment polarity genes revealed that head expression of *en* is controlled differently than trunk expression (DiNardo et al., 1988; Heemskerk et al., 1991). It is possible that vertebrates have maintained anterior functions of *en* and have not adapted it for a role in segmentation. Moreover, consistent with its role in vertebrate brain development, flies mosaic for *engrailed*-lethal cells show abnormalities in the brain (Lawrence and Johnston, 1984). It will be interesting to learn whether the genes that regulate anterior and neural *en* expression in flies are similar in nature to those that regulate vertebrate en genes.

The POU group

Comparison of sequences from several homeobox genes from mammals (*Pit-1*, *Oct-1*, *Oct-2*) and worms (*unc-86*) led to the discovery that in addition to highly conserved and distinctive homeoboxes, these genes contain another stretch of homology 5′ to the homeobox (reviewed in Rosenfeld, 1991). These two regions of homology and the linker region between them encode what is now referred to as the POU domain. Subsequently, many other genes encoding POU-domain proteins were cloned, including several from *Drosophila* (Johnson and Hirsh, 1990; Dick et al., 1991; Lloyd and Sakonju, 1991; Treacy et al., 1991; Treacy et al., 1992; Affolter et al., 1993). The mammalian POU genes have been grouped into 6 classes based on sequence similarities (Wegner et al., 1993).

In addition to the homeodomain, these proteins share an approximately 70 amino acid domain amino terminal to the homeodomain, the POU-specific domain, whose structure is discussed above. The POU-specific domain, located amino terminal to the homeodomain, is capable of binding DNA in the absence of the homeodomain, albeit poorly (Verrijzer et al., 1992). Thus, POU proteins contain two autonomous DNA-binding domains which work together to bind DNA avidly, with greater discriminatory capabilities. No gene identified to date contains a POU-specific domain in the absence of a homeodomain. The POU-specific domain in conjunction with the homeodomain is also required for homodimerization and heterodimerization of POU proteins (Ingraham et al., 1990; Voss et al., 1991; Verrijzer et al., 1992). The biological relevance of such interactions is unclear at this point, although in one case, heterodimerization of a *Drosophila* transactivator POU protein (*Cf1a*) with another POU factor (*I-POU*) renders the complex unable to bind DNA in vitro (Treacy et al., 1991). POU domain proteins contain a variety of transactivation domains and have been shown to activate transcription in co-transfection assays (Theill et al., 1989; Ingraham et al., 1990; Muller et al., 1990; Tanaka and Herr, 1990).

In mammals, many genes encoding POU domain proteins are expressed during early embryogenesis. In addition to their expression in the developing nerve cord, several POU domain proteins are expressed in many regions of the developing brain, including forebrain (for review, see Rubenstein and Puelles, 1994). None of the HOX genes are expressed in the forebrain, so POU domain proteins may play a crucial role in patterning events in this region of the CNS. To date, the only vertebrate POU gene mutant analyzed, *Pit-1*, has its late expression in the anterior pituitary (Li et al., 1990; Radovick et al., 1992; Tatsumi et al., 1992; Pfaffle et al., 1992). In these mutants, several cell types of the anterior pituitary fail to develop, suggesting that *Pit-1* is important for their specification and maintenance. *Pit-1* target genes include those encoding growth hormone and prolactin.

In *Drosophila*, several genes encoding POU domain proteins have been cloned (for review, see Wegner et al., 1993). The fly proteins fall into similar classes as the mammalian proteins and may share at least part of their function. For example, the fly genes similar to *Oct-1* and *Oct-2* (*dOct-1*, *dOct-2*) are expressed in the brain and neural tube of the fly, as are their mammalian counterparts. Three other fly POU genes (*Cf1a, I-POU, tI-POU*) and their mammalian relatives (*Brn-1*, *Brn-2*, *Brn-3.0*) are all expressed in nervous tissue.

The Pax group

Vertebrate *Pax* genes are distinguished by the presence of a paired box, encoding a 128 amino acid DNA-binding domain, that is found in *Drosophila* pair-rule and segment polarity genes including *paired*, *gooseberry* and *gooseberry distal* (Bopp et al., 1986). In addition, two fly genes have been isolated that contain a paired box but no homeobox. One of these (*Pox meso*) is expressed in mesoderm whereas the other (*Pox neuro*) is expressed in the nervous system (Bopp et al., 1989). This second class of paired genes, whose products are localized to the nucleus and may act as transcriptional regulators, appears to be under the control of *paired* and other segmentation genes.

So far, ten *Pax* genes have been identified in mammals (for reviews, see Chalepakis et al., 1993; Rubenstein and Puelles, 1994). Out of eight mouse *Pax* genes, half (*Pax-1, Pax-2, Pax-5* and *Pax-8*) do not contain homeoboxes. The ones that contain both homeoboxes and paired boxes (*Pax-3, Pax-6, Pax-7*) are expressed earlier than the others, similar to what is observed in flies. The *Pax-1* gene, which is expressed in the mesoderm, shows a very high degree of sequence homology (90%) with *Pox meso* in the paired domain (Bopp et al., 1989). All *Pax* genes except *Pax-1* are expressed primarily in the nervous system, including neural tube, neural crest cells and brain, in distinct anteroposterior as well as dorsoventral patterns. *Pax* expression is seen in other tissues, including excretory system, muscle, pituitary, pancreas, thyroid, B-cells, ear, eye, limb bud and testes (reviewed in Chalepakis et al., 1993). Whether *Pax* genes are involved in establishing polarity of the embryo, or control specific differentiation events, is unclear at this time.

To date, three mouse mutant phenotypes have been shown to be caused by mutations in *Pax* genes. Mice that bear the *undulated* mutation, which consists of a missense mutation in the Paired box of *Pax-1*, show defects in the axial skeleton (Balling et al., 1988). *Splotch* alleles, mutations in the *Pax-3* gene, cause central nervous system disorders such as excencephalus and spina bifida in addition to abnormalities associated with structures derived from the neural crest (Epstein et al., 1991). Mutation of the *Pax-6* locus (*small eye*) results in mice that lack eyes and nasal structures (Hill et al., 1991). Two human conditions resembling *Splotch* and *small eye*, Waardenburg Syndrome I and Aniridia, are caused by mutations in the human *PAX3* and *PAX6* genes, respectively (Baldwin et al., 1992; Tassabehji et al., 1992). *PAX3* has also been implicated in human rhabdomyosarcomas (Shapiro et al., 1993). In an astonishing example of conservation, the gene *eyeless* of *Drosophila* has been found to encode a protein similar to *PAX6* (Quiring et al., 1994). Thus, *Pax-6* appears to have become dedicated to the visual part of the brain prior to the separation of vertebrate and invertebrate lineages. The strikingly distinct eye structures in mammals and insects apparently conceal a startling common ancestry, much as in the case of the insect and vertebrate hearts.

CONSERVATION UPSTREAM: ZINC FINGER PROTEINS AND LIGAND DEPENDENCE

In both vertebrates and insects, some mechanism must couple maternal or other positional information to the localized activation of the Hox genes. It is still not clear what if any components of this mechanism are similar in these two groups of animals. The apparently vast differences between the syncytial beginning of *Drosophila* embryogenesis and the formation of the inner cell mass in mammals suggests distinct mechanisms must exist, yet the outcome in both cases is differential anterior-posterior Hox expression. We face two mysteries: how Hox genes are regulated and how their different forms of regulation evolved.

There are few mammalian regulators of Hox genes known. The most intensively studied ones are retinoic acid (controlling transcription through its zinc finger protein receptor), the *Krox20* zinc finger protein and the *Sonic hedgehog* (*Shh*) signalling protein. Retinoic acid (RA) regulates Hox genes in cultured cells: the Hox genes are sequentially induced with time or with increased RA concentration (Mavilio et al., 1988). Elegant studies of *Krox20* regulation of *Hoxb2* in the hindbrain rhombomeres has established the high probability of a direct interaction with defined control elements (Sham et al., 1993). *Shh* activates Hox genes by acting as an apparent morphogen in limb development (Riddle et al., 1993). The *Shh* protein is a signalling protein and its receptor is unknown.

The regulation of vertebrate Hox genes by RA receptors and *Krox20* is reminiscent of some of the regulators of Hox gene expression in flies. The 'gap' segmentation genes have been found to regulate initiation of homeotic gene transcription (Harding and Levine, 1988; Irish et al., 1989). Gap genes encode zinc finger proteins, at least two of which, *knirps* and *tailless* proteins, are similar to steroid hormone receptors (Jäckle et al., 1985; Pignoni et al., 1990). There are no known ligands for the gap gene proteins. However, it is intriguing to suppose that ligands may be necessary in animals which, unlike *Drosophila*, do not pass through a syncytial stage of development. If the ligands are able to cross membranes they may function as RA may in mammals. Signalling molecules may govern transcription of Hox genes in both cases.

MAINTENANCE OF REPRESSION

Many of the regulators of homeotic genes are expressed early in *Drosophila* development and then disappear. Yet the genes continue to be spatially regulated. Some progress has been made on understanding genes involved in the maintenance of active or repressed gene states, the genome expression memory systems. Very little is yet known of the extent to which similar systems exist in other animals. A process as fundamental as gene expression maintenance seems likely to be conserved (although animals differ greatly in the degree to which they methylate DNA).

Regulators of homeotic gene transcription have been grouped according to their negative or positive effects.

Members of the *Polycomb* group (Pc-G) are negative regulators required to keep homeotic genes inactive where they should be inactive (reviewed in Paro, 1993). Conversely, members of the trithorax group (Tr-G) have activating functions necessary to maintain homeotic transcription after it has been initiated (reviewed in Kennison, 1993). While these generalities are a good starting guide, the individual properties of many members of both groups have not been fully explored and it is likely that the grouping into positive and negative regulators masks unique attributes of the loci.

The view that Pc-G genes are involved in maintenance of homeotic gene expression is based upon experiments in which homeotic gene transcription was monitored in Pc-G mutants. Initial patterns were normal, but later transcripts were observed where they are not normally found (Struhl and Akam, 1985; Wedeen et al., 1986).

Protein products of three of the Pc-G genes have been localized to about 100 sites on the salivary gland polytene chromosomes (Zink and Paro, 1989; Zink et al., 1991; DeCamillis et al., 1992; Martin and Adler, 1993). The colocalization of the three negative regulators *Polycomb*, *Posterior sex combs* and *polyhomeotic* suggests a very close functional relationship, and indeed evidence has been obtained for a biochemically detectable protein complex containing *Pc* and *ph* proteins (Franke et al., 1992). The *Polycomb* protein contains a short sequence similar to a part of the *Drosophila* HP-1 heterochromatin protein, termed the chromodomain (Paro and Hogness, 1991). Proteins containing this domain have also been observed in vertebrates (Singh et al., 1991; Pearce et al., 1992). Beyond strengthening the hypothesis that Pc-G proteins repress through a mechanism related to heterochromatin-mediated inactivation, the chromodomain has been shown to be sufficient for chromosome localization at the proper 100 sites. Nothing is known about the degree to which *Polycomb* is functionally conserved in vertebrates.

A second case of conservation has also been found, involving the two Pc-G members *Su(z)2* and *Posterior sex combs* (*Psc*). Proteins coded by these two genes share similar regions with the *bmi-1* mammalian proto-oncogene (Brunk et al., 1991; van Lohuizen et al., 1991). Part of the similar region may be a novel type of zinc finger. The regions of similarity are dispersed through much of the proteins, suggesting conservation of a large domain or multiple domains.

HRX, TRX AND LEUKEMIA

The *trithorax* (*trx*) gene in *Drosophila*, originally called *Regulator of bithorax*, is responsible for appropriate activation of Antennapedia and bithorax complex homeotic genes (Ingham and Whittle, 1980; Capdevila and Garcia-Bellido, 1981). Thus, *trx* mutant flies show homeotic transformations due to insufficient production of Hox proteins. The maintenance of expression of certain homeotic genes, eg *Ubx*, is more sensitive to the loss of *trx* than others such as *Antp* (Breen and Harte, 1993). Mutations in Polycomb group genes are suppressed by *trx* alleles (Ingham, 1983). Thus, reduced repressor function is balanced by reduced activator function. The *trx* gene has been cloned and shown to encode a very large protein (3759 amino acids) containing many cysteine-rich zinc finger-like domains which are found in proteins that bind DNA (Mazo et al., 1990).

The cloning of a gene involved in translocations associated with acute leukemias led to the discovery that it encodes a human *trx* homolog (*Hrx*; Djabali et al., 1992; Gu et al., 1992; Tkachuk et al., 1992). The encoded protein is 3968 amino acids in length and contains several zinc finger domains. The human protein contains several domains similar to DNA-binding AT hook motifs originally identified in HMG proteins, which are associated with active chromatin structures (Tkachuk et al., 1992). Three regions show homology to the fly *trx* protein, including a carboxy terminal domain that is 82% similar with 61% identity. *Hrx* is expressed during fetal development (Gu et al., 1992).

UPSTREAM ACTIVATORS? THE SWI/SNF COMPLEX AND *BRAHMA*

brahma is another activator of homeotic gene transcription (Kennison and Tamkun, 1988; Tamkun et al., 1992). The encoded protein is similar to a yeast protein called SWI2 or SNF2, whose roles in regulating transcription are reviewed in Winston and Carlson (1992). SWI2, which encodes an ATPase (Laurent et al., 1993), is a member of a 2×10^6 M_r complex containing about ten proteins (Cairns et al., 1994; Peterson et al., 1994). Flies and mammals may contain a SWI/SNF complex of similar size (Khavari et al., 1993; J. W. Tamkun, personal communication; G. R. Crabtree, personal communication). The yeast complex is thought to activate a subset of genes, possibly by opposing the repressive effects of chromatin (Peterson and Herskowitz, 1992). Proteins related to *brahma* have been identified in mammals, though their functions in vivo are unknown (Okabe et al., 1992; Soininen et al., 1992; Khavari et al., 1993; Muchardt and Yaniv, 1993).

COLLABORATORS: THE *EXD/PBX* GROUP

The fly gene *extradenticle* (*exd*) may encode a cofactor that can work together with Hox transcription factors. *exd* mutations result in homeotic transformations of body segments, even though the homeotic genes are expressed at the appropriate times and places (Peifer and Wieschaus, 1990). The cloning of *exd* led to the exciting discovery that the *exd* gene encodes a homeodomain-containing protein with significant similarity to the products of two different genes, the yeast transcriptional repressor MAT**a**1 (which itself is a cofactor for the homeodomain-containing MATα2 protein) and the human PBX homeobox gene family consisting of *PBX-1*, *PBX-2* and *PBX-3*. In yeast, the MAT**a**1/MATα2 heterodimer, which is responsible for repressing haploid-specific genes, binds a different set of target sites than the MATα2 homodimer, which represses **a**1-specific genes. Similarly, the combined action of *exd* and the homeotic gene products could determine binding site selectivity. This may explain the lack of binding site discrimination observed for many homeodomain proteins in in vitro DNA binding assays.

Recently, it has been shown that the proper expression patterns of three target genes regulated by fly homeotic genes (*dpp, wg, tsh;* see below) require *exd* function (Rauskolb and Wieschaus, 1994). The normalcy of Hox gene expression and the altered expression of the target genes regulated by Hox

proteins is consistent with a model of *exd* protein as a cofactor of some sort.

PBX-1, one of three *exd*-like genes known in mammals (Monica et al., 1991), was cloned based on its involvement in chromosomal translocations associated with acute leukemias (Kamps et al., 1990; Nourse et al., 1990). One translocation results in the fusion of a portion of PBX-1 including its homeobox with a portion of a helix-loop-helix gene (E2A) with homologies to the *Drosophila daughterless* gene. This gene fusion was shown to be under the control of the E2A regulatory sequences. Further analysis has shown that the E2A/PBX fusion is necessary and sufficient to produce tumors when expressed in blood cells in the mouse(Dedera et al., 1993). The E2A region of the fusion contains a transcriptional activation domain and may be imparting on an otherwise 'inactive' PBX protein a transactivation capability. Since PBX does not activate transcription in co-transfection assays, it is intriguing to speculate that PBX must interact with other factors, perhaps Hox products, to regulate transcription of its target genes. Fusion of an activation domain to PBX might result in inappropriate regulation of these target genes.

INFORMATION FLOWING DOWNSTREAM: ARE TARGETS CONSERVED?

The *Dfd* autoregulatory element is a well-characterized homeotic regulatory element (HRE). *Dfd* protein can activate the element and requires the homeodomain-binding sites within it to do so (Kuziora and McGinnis, 1988; Bergson and McGinnis, 1990; Regulski et al., 1991). Remarkably, the autoregulatory element may be conserved in function to mice, as an element from a mouse *Dfd*-like gene gives appropriate head-specific expression in response to *Dfd* in flies (Malicki et al., 1992). Conversely, the fly element drives localized expression in mouse brain (Awgulewitsch and Jacobs, 1992).

Few direct (or probably direct) target genes of Hox proteins have been identified (reviewed in White et al., 1992; Botas, 1993). The cross-regulatory interactions observed between fly Hox genes have not as yet been seen in mammals. The list of other target genes in flies is short and includes: *decapentaplegic* (*dpp*) and *wingless* in the midgut (Immerglück et al., 1990; Reuter et al., 1990; Capovilla et al., 1994), *teashirt* in the epidermis (Röder et al., 1992) and midgut (Mathies et al., 1994), *spalt* in imaginal discs (Wagner-Bernholz et al., 1991), *connectin* in neuromuscular tissue (Gould and White, 1992), *ems* in the epidermis (Jones and McGinnis, 1993) and *Distal-less* in leg primordia (Vachon et al., 1992). The last two cases mentioned may represent a type of cross-regulation if these genes were originally members of an ancestral Hox cluster.

Virtually nothing is known about target genes in mammals. An intriguing parallel is seen, however, in midgut development of flies and mice (Roberts et al., unpublished data). In flies the transcription of *dpp*, which encodes a TGFβ-class secreted protein, is activated by the *Ubx* homeotic protein in the visceral mesoderm. The two vertebrate proteins most similar to the *dpp* protein are bone morphogenetic proteins 2 and 4 (BMP2, 4; Jones et al., 1991). BMP2 is expressed in the visceral mesoderm of the chick in a region of the gut where homeotic genes are also expressed. The distinct, non-overlapping domains of homeotic gene expression in the *Drosophila* midgut are reminiscent of the discrete domains of Hox expression in the chick midgut. The boundaries of Hox gene expression correspond to the boundaries between different tissue types in the gut, suggesting regulation of gut differentiation by Hox genes may be common to insects and mammals, and may even involve some of the same target genes. The most direct evidence for a role of Hox genes in gut differentiation comes from the phenotype of ectopically expressed *Hoxc-8*, which causes defects in stomach differentiation (Pollock et al., 1992).

CONCLUSIONS

We confront a remarkable array of conserved regulators of development. In some cases, but probably not all, the conservation goes beyond protein structure to conservation of the relationships between types of molecules and the parts of an animal that they control. In many cases the proteins are needed in a variety of tissues and cannot be viewed as dedicated to one organ or tissue. Functions common to many organisms may identify the original sites of gene action. Two questions of outstanding importance are: to what extent did proteins become dedicated to particular developmental processes more than half a billion years ago, and why? What special features of regulators allow them to play their roles? The tools to approach these questions are in hand and we can look forward to new views of developmental regulation when the next decade of homeobox research has passed.

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